THE UREIDES OF *p*-AMINOPHENYLSTIBINIC ACID

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W. H. GRAY, J. W. TREVAN, H. W. BAINBRIDGE AND A. P. ATTWOOD

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THE WELLCOME CHEMICAL RESEARCH LABORATORIES
(The Wellcome Foundation Ltd.)
T. A. HENRY, D.Sc., *Director*
6, King Street, Snow Hill
LONDON, E.C.1

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By W. H. GRAY, Wellcome Chemical Research Laboratories; J. W. TREVAN, H. W. BAINBRIDGE, and A. P. ATTWOOD, Wellcome Physiological Research Laboratories.*

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1. Indian kala-azar is a widespread disease which ran a fatal course, uninfluenced by drugs, until it was found that it could be cured by tartar emetic (Di Cristina and Caronia, 1915; Rogers, 1915; Muir, 1915). More recently it has been shown by a number of workers that certain organic antimony compounds can be used with more advantage. These are far less toxic in proportion to their curative power, and are thus suitable for intensive administration. Several of the more important are simple derivatives of *p*-aminophenylstibinic acid (stibanilic acid), of known constitution, for example, the sodium salts of the glucosyl-(stibamine glucoside) and the 3-chloro-4-acetyl-derivatives, also the diethylamine salt of the acid itself. Apart from these, the most interesting is a material prepared by heating stibanilic acid with urea solution, introduced by Brahmachari (1922) under the name "urea stibamine," the nature of which has been the subject of conflicting opinions by other workers as well as by Brahmachari himself. As this substance is in extended use in India, and it is important from the point of view of progress in chemotherapy that the constitution of useful drugs should not remain undetermined, the present investigation, with the initial object of elucidating this, was undertaken.

2. At the outset the product was found to be, not ammonium *p*-carbamidophenylstibinate, which is Brahmachari's latest view (1924), but a somewhat complicated mixture of colloids. Only partial separation of its constituents could be effected by chemical means, which, however, sufficed to show that this ammonium salt could not be present to any considerable extent, and to indicate the presence of the essential active substance with respect to mouse trypanosomiasis (*Trypanosoma equiperdum*), *s*-diphenylcarbamide-4 : 4′-

* W. H. Gray is responsible for the chemical section, and J. W. Trevan, H. W. Bainbridge and A. P. Attwood, by whom all the experiments on animals were made, for the physiological.

distibinic acid. Verification of this was afforded by preparation of these two ureides by separate and simpler methods and comparison of their physiological properties with those of "urea stibamine (Brahmachari)."

3. The latter substance is prepared by heating stibanilic acid with urea solution, this acid being obtained by the customary alkaline hydrolysis of its acetyl-derivative. It is now found that the published directions for the latter process are insufficiently definite in one important particular, viz., the method of determining when hydrolysis is complete. Products containing varying amounts of unchanged *p*-acetylaminophenylstibinic acid may be obtained according to the conditions chosen for this test, still keeping within the scope of these published directions. When such a product is treated, under suitable conditions, with urea, the stibanilic acid present reacts in two ways :—

(*a*) With formation of the ureide.

(*b*) Undergoing hydrolytic fission with formation of antimonic acid.

The former, in a pure state, does not form appreciably soluble salts ; its solubility in presence of the antimonic and acetylaminophenylstibinic acids is to be attributed to the action of these substances as protective colloids (see § 9). The relative amounts of the three substances in the final product, as well as of urea, which is difficult to remove from these amorphous materials, depend on the experimental conditions. This source of variation accounts for the difficulty in repeating the preparation of "urea stibamine" which has been reported by other investigators (Napier, 1923 ; Uhlenhuth, Kuhn and Schmidt, 1925), and possibly for the alleged variation in the composition of the samples on the Indian market (Ghosh and others, 1928 ; Napier, 1929).

4. It will be seen from the foregoing that this problem has been investigated biologically as well as chemically, and the opportunity has been taken to include in the physiological section, on a basis of the results obtained, a general discussion of methods appropriate for the examination of all such chemotherapeutic problems from the physiological standpoint.

5. There is no evidence that the relative values of these products, as estimated by these experiments on mice, are transferable to their use in human kala-azar. For example, we find that stibamine glucoside is less active on trypanosomiasis in the mouse than "urea stibamine," whereas Napier (1929) finds that the two drugs are equal in therapeutic efficacy for kala-azar.

Chemical Section.

6. " Urea stibamine (Brahmachari) " was analysed and gave the following results :—

Table I.

Batch No.	Loss on drying.	C.	H.	N.	Sb.	Atomic ratios.	
						C : N.	C : Sb.
425	10·38	—	—	6·75	44·19	—	—
425	10·38	—	—	6·77	44·49	—	—
451	7·14	20·2	3·0	—	—	—	—
522	12·17	20·9	2·9	—	—	—	—
522	14·07	20·5	2·8	—	—	—	—
522	12·17	20·9	3·0	—	46·4	—	4·57 : 1
522	16·27	21·53	2·67	—	—	—	—
926	10·12	21·16	2·8	—	46·8	—	4·59 : 1
1128	9·45	20·17	2·91	6·47	48·6	3·64 : 1	4·21 : 1

The value found for the ratio C : N shows that the product cannot be ammonium *p*-carbamidophenylstibinate, which requires 2·3 : 1 ; the value of the ratio C : Sb shows that it cannot consist of a pure arylstibinate, but must also contain antimony in inorganic combination. This is shown to be antimonic acid (see § 9). In order that a determination of the ratio C : N should afford trustworthy information as to the nature of the organometallic constituent, the product was treated with dilute hydrochloric acid to remove ammonia or urea. The resulting solid contained C, 20·2 ; H. 2·4 ; N, 3·4 ; Sb, 52·2 per cent., atomic ratio C : N = 6·98 : 1, showing that the trypanocidally active substance is not *p*-carbamidophenylstibinic acid, which requires C : N = 3·5 : 1. Further information was given by a prolonged treatment of " urea stibamine " (4 g.) with dry alcohol, when the portion remaining undissolved (2·95 g.) was found to have the same composition as the solid obtained by the action of dilute acid (C, 20·5 ; H, 3·1 ; N, 3·5 ; Sb, 50·0 per cent., atomic ratio C : N = 6·91 : 1). The difference in the total nitrogen before and after extraction with alcohol consequently represents the ammonia originally present, removed by the alcohol and volatilised during the evaporation. Calculated on the basis of the total stibinic acid present, this corresponds to approximately 0·36 of a molecular proportion. The residue from the extracts (0·51 g.) contained urea (3·7 per cent. by weight of the original material*)

* The statement of Ghosh and others (1928) that " most of the free urea can be washed out in the cold " is therefore not correct.

and a stibinic acid portion of low trypanocidal activity, probably containing acetylaminophenylstibinic acid (see § 10), which was not obtained in quantity sufficient for identification. The undissolved portion was more active than " urea stibamine " itself, and therefore contained the trypanocidally-active constituent.

7. The alternative remaining is that the latter consists of the di-substituted urea, *s*-diphenylcarbamide-4 : 4′-distibinic acid, which requires an atomic ratio of C : N = 6·5 : 1. The formation of this under the conditions of the preparation was seen to be quite feasible, as it had been shown by Young and Clark (1898) that a mono-substituted urea is converted into the corresponding symmetrical di-substituted urea by boiling with water. The correctness of this conclusion was confirmed in two ways :—

(*a*) In investigating the action of urea upon *p*-aminophenylstibinic acid, the relative amounts of urea and water, the temperature, and the time of heating, were found to have a marked influence on the composition of the solid product precipitated by alcohol. These factors were not specified by Brahmachari, nor adequately by the other workers who criticised his directions as lacking in experimental detail (Ghosh and others, 1928 ; Niyogi, 1928). When the concentration of the urea was kept low, in order to favour the formation of a di-substituted urea, as in § 8 and § 10, the product had a trypanocidal action equal to or somewhat greater than that of " urea stibamine," whilst a high concentration of urea led to the formation of a comparatively inactive material. Further, the atomic ratios of carbon to nitrogen in these products, after purification by dilute hydrochloric acid, were 6·5 : 1 and 3·4 : 1 respectively, corresponding in the one case to the presence of *s*-diphenylcarbamide-4 : 4′-distibinic acid as organometallic constituent, in the other of *p*-carbamidophenylstibinic acid.

(*b*) These two ureides have been synthesised, and the former shown to have the greater trypanocidal activity, although, owing to the fact that it does not form soluble salts and consequently must be administered in suspension, intraperitoneally instead of intravenously, it is not quite so active as might be expected.

8. *Preparation of a Product containing* s-*Diphenylcarbamide*-4 : 4′-*distibinic Acid as Trypanocidal Constituent, by means of Urea.*—A paste of freshly-precipitated *p*-aminophenylstibinic acid, containing 13 g. of water, prepared by hydrolysis of the free acid from 10 g. of stibacetin, using 10 per cent.

hydrochloric acid to test the hydrolysing solution, was treated with solid urea (4·2 g. = 3 molecular proportions), the mixture heated at 80° with continuous stirring until a drop no longer gave a precipitate on dilution with water, allowed to stand overnight and treated with alcohol (100 c.c.). The resulting solid required the addition of a little ammonia for solution, and was dissolved in ammonia and reprecipitated by alcohol, after which it was soluble in water. Yield, 5·03 g. (found : C, 16·9 ; H, 2·8 ; N, 3·7 ; Sb, 45·6 per cent.). The trypanocidal action of this was somewhat greater than that of "urea stibamine," and both this and the composition were reproducible (key numbers of batches physiologically tested, G.10 and G.11). Treatment with dilute acid (see § 6) gave a solid containing C, 14·5 ; H, 2·3 ; N, 2·6 per cent., atomic ratio C : N = 6·5 : 1).

9. The low carbon content in this (required, C 28·2) and in the other cases mentioned in this paper, is due to the presence of antimonic acid. This is shown by the fact that the mother liquors contain aniline, which must have been formed by hydrolysis of the carbon-antimony linkage. In a control experiment, it was found that, in the presence of *p*-aminophenylstibinic acid, antimonic acid was reversibly soluble in ammonia. The formation of reversibly soluble protected antimonic acid in this way does not seem to have been described, although both antimonic acid (Jander, 1918) and the arylstibinic acids (Schmidt, 1920) are known to be pronouncedly colloidal. It is somewhat surprising that this fission occurs also to a considerable extent in the cold. A paste of freshly-precipitated *p*-aminophenylstibinic acid containing an equal weight of water was mixed with solid urea (12 molecular proportions). Rapid solution of both solids took place, and treatment with dilute hydrochloric acid gave, not the expected solution of hydrochlorides but a solid, in which the carbon content had fallen from 25 to 19 per cent., with solubility properties simulating those of the "free acid" obtained from "urea stibamine." Here, rearrangement of the urea cannot have occurred (Werner, 1913), so that it is incorrect to assume, as Brahmachari (1924) has done, that insolubility in dilute hydrochloric acid, in this series, indicates the absence of a free amino-group. This was confirmed by the fact that the ratio C : N remained unchanged in this case.

10. *Preparation of a Product Identical with "Urea Stibamine (Brahmachari)."*—The above product shows small differences from the commercial material (*a*) in the C : N ratio of the organometallic constituent, (*b*) in solubility in water, (*c*) in trypanocidal activity, (*d*) in the amount of antimonic acid present. This difficulty was overcome when it was found that, by suitable

choice of concentration of hydrochloric acid in the test for the point of completion of hydrolysis of the acetylaminophenylstibinic acid, which is not specified in the published methods (Chem. Fabr. von Heyden, 1912 ; Schmidt, 1922), a certain mixture of aminophenylstibinic acid and acetylaminophenylstibinic acid was obtained which still fulfilled the conditions of this test, and yielded on suitable treatment with urea a product identical with the commercial material in all these respects. The C : N ratio of 7 : 1 is accounted for by the presence of unchanged acetylaminophenylstibinic acid (C : N = 8 : 1) ; this helps to preserve the solubility of the mixture of weak acids by preventing loss of ammonia on drying. The free acid from 15 g. of stibacetin was hydrolysed, using decinormal hydrochloric acid to test for completion. In a control preparation, the product so obtained was found to contain C, 28·3 ; H. 3·2 ; N, 4·7 per cent., atomic ratio C : N=7·02 : 1. The freshly precipitated product was uniformly suspended in water (916 c.c.) treated with urea (20·6 g. = 10 molecular proportions) and heated at 80° with continuous stirring until a clear solution was obtained (2 hours), allowed to stand overnight, concentrated to 40 c.c. in a vacuum, and treated with dry alcohol (350 c.c.). The solid was dissolved in a little water and precipitated with dry alcohol. Yield, 4·9 g. ; readily soluble in water to a very faintly acid solution, resembling that of "urea stibamine (Brahmachari)" ; (found : C, 20·7 ; H. 3·4 ; N. 4·4 ; Sb, 48·4 per cent.). Key number G.12. The nitrogen content shows that it contained a little less impurity of urea than the latter, but purification with dilute hydrochloric acid (see § 6) gave a product of the same composition in each case (found : C, 18·3 ; N, 3·0 per cent., atomic ratio C : N = 7·07 : 1).

11. *The Action of Urea upon* p-*Acetylaminophenylstibinic Acid.*—A control experiment to ascertain the fate of this acid under the conditions of Brahmachari's preparation, since it is a constituent of the starting material used in § 10, showed that it gradually dissolved, yielding a water-soluble product* which, after the purification with dilute hydrochloric acid described in § 6, contained C, 27·1 ; H. 3·1 ; N, 4·3 per cent., atomic ratio C : N = 7·33 : 1. This value for the ratio shows that the soluble material was not pure ammonium *p*-acetylaminophenylstibinate but that a certain amount of de-acetylation

* Brahmachari and Das Gupta (1929) have since stated that urea *p*-acetylaminophenylstibinate is formed when the acid is heated with urea. The properties of the product are not given ; the behaviour of *p*-acetylaminophenylstibinic acid, described above, precludes this assumption, which is also seen to be improbable from their method of preparation, which involves heating to 100°.

had occurred. Whether this was followed by the formation of the N-acetyl-derivative of *s*-diphenylcarbamide-4 : 4′-distibinic acid was not determined ; the product was, however, of low activity, and therefore not of consequence as a trypanocidal constituent of " urea stibamine."

12. s-*Diphenylcarbamide*-4 : 4′-*distibinic Acid.*—Stibacetin (9·7 g.) was hydrolysed as in § 8. The alkaline hydrolysed solution was almost neutralised by 2N-hydrochloric acid and treated with half-saturated sodium acetate solution (78 c.c.). A 12·5 per cent. solution of phosgene in toluene (45 c.c.) was then added in three portions, shaking well, and the mixture shaken for several hours. It was then acidified with 20 per cent. hydrochloric acid (50 c.c.) and the solid was washed with 1·2 per cent. hydrochloric acid, yield 6·5 g. It is not appreciably soluble in alkalies or the ordinary organic solvents (found : C, 28·1 ; H. 2·8 ; N. 4·8 ; Sb, 44·6 ; $C_{13}H_{14}O_7N_2Sb_2$ requires C, 28·2 ; H. 2·5 ; N. 5·1 ; Sb, 44·0 per cent.). Key number G.9. Treatment with cold concentrated hydrochloric acid led to the formation of a substance with the properties of the corresponding stibine tetrachloride, and approximating in composition to this.

13. *Preparation of a Product containing* p-*Carbamidophenylstibinic Acid as Trypanocidal Constituent, by means of Urea.*—A paste of *p*-aminophenylstibinic acid containing 20 g. of water, from 15 g. of stibacetin, was well mixed with solid urea (45 g. = 22 molecular proportions), and the resulting solution heated for 7 hours at 75°. It was then treated with dry alcohol (270 c.c.). The solid was treated with alcohol until unchanged in weight (6·4 g.) (found : C, 13·9 ; H, 2·8 ; N, 4·8 ; Sb, 52·3 per cent.). The trypanocidal activity of this is considerably less than that of the corresponding preparation of the di-substituted urea, § 8, making allowance for the fact that fission was somewhat more extensive under the conditions of this preparation (key number G.7). Treatment with dilute acid (see § 6) gave in this case a solid containing C, 10·9 ; H, 1·8 ; N, 3·7 per cent., atomic ratio C : N = 3·4 : 1.

14. p-*Carbamidophenylstibinic Acid.**—Freshly-precipitated *p*-aminophenylstibinic acid from 7 g. of stibacetin was dissolved in sodium hydroxide to a bicarbonate-alkaline solution, the sodium salt precipitated by acetone and dried at room temperature until the acetone was just removed. To this

* Whilst this manuscript was in course of preparation two papers appeared : by Niyogi (1928), stating that ammonium *p*-carbamidophenylstibinate and " urea stibamine " are identical, and by Schmidt (1930) stating that they are different in properties, and that Roehl found ammonium *p*-carbamidophenylstibinate to be inactive in kala-azar in the hamster.

was added 4 g. of a fresh sample of potassium cyanate, and water until the volume was 30 c.c. After being clarified it was treated with glacial acetic acid (4 c.c.) allowed to stand 1 hour, diluted with water (40 c.c.), treated with 25 per cent. hydrochloric acid (13 c.c.), filtered, and washed with 1·2 per cent. hydrochloric acid. Yield, 4·3 g. (found : C, 27·1 ; H, 2·8 ; N, 8·7 ; Sb, 42·3 ; $C_7H_9O_4N_2Sb$ requires C, 27·4 ; H. 3·0 ; N, 9·1 ; Sb, 39·7 per cent.). The *sodium salt* was readily soluble in water (key number G.5). Although insoluble in cold ammonia, when precipitated together with antimonic acid this acid gave a reversibly-soluble product.

15. The possibility was visualised of the occurrence, simultaneously with the process of hydrolytic fission of the carbon-antimony linkage described in § 9, of the addition of the elements of water in the reverse order. Since, in this case, antimonious acid would also be formed, the question was investigated by treatment of the various products, described in this paper, with iodine in presence of excess of sodium bicarbonate in the cold. A negligible absorption took place, no greater than in the case of the stibacetin used as starting material. *p*-Aminophenylstibinic acid, on the contrary, reacted with approximately a molecular proportion of iodine, with formation of *p*-iodoaniline. Although this acid is known to yield *p*-iodoaniline when heated with hydriodic acid (Chem. Fabr. von Heyden, 1912) the formation of this in alkaline solution in the cold is unexpected, in view of the greater stability of the linkage of carbon to antimony under these conditions.

Physiological Section.

A.—*Therapeutic Action.*

16. *Methods.*—The therapeutic activity of these compounds was compared in the following way. Mice of 10 to 15 grams weight were infected intraperitoneally with *Trypanosoma equiperdum* (the strain used in this country for standardising the organic arsenicals). About 0·1 c.c. of an approximately 1/50 suspension of the blood of a mouse infected about 3 days before from a guinea-pig carrier was used. The mice were bred in the laboratory and were divided into two groups, so that each mouse in one group had a litter mate in the other group, the number of litter mates in each group being approximately equal. The drugs were injected into the tail vein, one group receiving the drug being examined and the other group receiving, in each experiment, a dose of stibamine glucoside. As a standard of reference in these experiments it was desirable to use a substance belonging to the same class, that is to say, an

organic antimony compound rather than one of the antimonyl tartrates, since the latter have toxic effects of a particular kind which are now supposed to be due to separation of antimony trioxide by the serum alkali. "Neostam" (the nitrogen-glucoside of sodium *p*-aminophenylstibinate) was selected for the purpose, being available in quantity and of constant physiological properties.

17. In the earlier experiments the injection was made at the time when the number of trypanosomes in the blood was about 500,000 per cubic millimetre, but in the course of the investigation the rate of development of infection became so fast that we lost mice by waiting an extra day, and we therefore gave the injection earlier when the infection was just appearing in the blood. No significant difference appears, except in so far as with very heavily infected mice no dose of drug will remove the trypanosomes. Care was taken not to use for infection any strain of trypanosomes which might have developed resistance to antimony by exposure to the action of an antimonial preparation. As with most of the organic arsenic compounds, the action of these drugs on this infection is only temporary, the trypanosomes almost invariably re-appearing in the blood in the course of some days.

18. The degree of infection was judged by microscopic examination of a drop of undiluted blood squeezed between a slide and a coverslip sufficient to cover about one-eighth area of a $\frac{3}{4}$-inch coverslip, using an 8-mm. apochromat objective and an 18 eye-piece. Arbitrary values expressed by the number of + signs were given to indicate the degree of infection, + representing three to four in twenty fields, ++ representing one trypanosome per field, and 4+ (++++) the condition when the trypanosomes appeared to be as numerous as the corpuscles and number about 2,000,000 per cubic millimetre. It is essential to use a fairly large number of animals to obtain an estimate of the variation in result between different individuals and it was impossible to count trypanosomes in every animal. Our impression is that the estimate by eye does not introduce an error comparable in dimensions with the variation between different animals. We are convinced that the variability in the comparative curative action of any one of these drugs from one animal to another is only slightly dependent on the degree of infection at the time within fairly wide limits; and, if each test is made in the form of a comparison with a standard, the conditions are similar for both the drugs and the standard of comparison.

19. Some considerations as to factors affecting the accuracy of the method are given in the discussion. There was, however, more tendency in certain of the experiments, where the injection of the drug was made before any trypano-

somes appeared in the blood, for an odd mouse to remain alive without trypanosomes for much longer than any of its fellows in the same group, although, even when the infection was advanced at the time of treatment the same might occur, but more rarely. These odd mice considerably diminish the statistical significance of the difference between groups of mice under comparison, by increasing the calculated standard deviation ; and if any way can be found to eliminate them, a considerable increase in the accuracy of comparison is likely. It is possible that the development of some degree of immunity by the mice is responsible for these happenings, and it is not difficult to imagine, from what is known of the precipitate development of immunity to other organisms after a long and variable latent period, that a very slight difference in the conditions for one mouse in a group might make a relatively large difference in its immunity response.

Experimental Therapeutic Results.

20. *Example of Experiments.*—One experiment on the therapeutic comparison will be described with full protocols, but for the rest of the experiments only a shortened account will be given.

Experiment 1.—Two groups of mice, 8 and 10 in number, which were made up of litter mates, were infected with diluted blood from a mouse heavily infected with trypanosomes. The following day one plus (+) was found in the blood of all except five mice, and all were injected. The group of 8 was injected intravenously with "urea stibamine (Brahmachari)" (B.926), the dose being 1·5 mg. per 20 g. of mouse, the weight of the animals varying from 10 to 16 g. ; the group of 10 was injected with 3·0 mg. of stibamine glucoside per 20 g. of mouse. The blood was observed each day until all the animals were dead. The history of each mouse in this experiment is given in Table II. The table shows the degree of infection of each mouse, identified by its own number throughout the table, on and after the day of injection of the drug. It will be seen that those animals which showed no trypanosomes on the day on which the drug was injected did not depart in rate of re-infection beyond the range of variation of the other animals.

Table II.—Intensity of Infection in Two Groups of Mice. Mice Nos. 1 to 8 were given 1·5 mg. of urea stibamine B.926; 9 to 18, 3·0 mg. of stibamine glucoside for 20 g. mouse. The first figure for each mouse is the number of plus signs given on the day of injection, the succeeding figures the number of plus signs for each successive day till death (D).

Mouse No.	1	1	0	0	0	0	0	0	0	0	0	0	0	1	2	2	D				
,,	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4	D
,,	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	3	4	D				
,,	4	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	4	D		
,,	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	3	4	D		
,,	6	0	0	0	0	0	0	0	0	0	0	0	0	1	3	4	D					
,,	7	0	0	0	0	0	0	0	0	0	0	1	1	2	3	D						
,,	8	1	0	0	0	0	0	0	0	0	0	0	0	1	2	2	D					
,,	9	1	1	0	0	0	0	0	1	3	4	D										
,,	10	1	0	0	0	0	0	1	2	4	4	D										
,,	11	1	0	0	0	0	0	0	0	0	0	0	0	1	3	D						
,,	12	2	0	0	0	0	0	0	0	0	0	0	0	0	0	4	D					
,,	13	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2	3	D	
,,	14	1	0	1	0	0	0	0	1	1	4	4	D									
,,	15	0	0	0	0	0	0	0	1	1	3	4	D									
,,	16	0	0	0	0	0	0	0	0	0	0	2	4	4	D							
,,	17	1	0	0	0	0	0	0	1	3	D											
,,	18	1	0	0	0	0	0	0	0	0	3	4	D									

21. The difference between the action of the two drugs can be expressed in three different ways, namely, (1) the average time which the mice survived; (2) the average number of days on which no trypanosomes were found in the blood; (3) by a figure which measures, not only those days on which the mice had no trypanosomes in their blood, but also those days on which the trypanosomes were present in varying degrees. This is obtained in the following manner, and we have called it the "total therapeutic effect." For each individual mouse a score is calculated by subtracting the number of plus signs for each day from 5, 5 plus signs being assigned to the day on which the animal was found dead. These figures are added up for each mouse and the average obtained. The means for all three methods are given in Table III, column 4, which shows that in this disease, "urea stibamine," in this dose, gives mean values of (1) survival time, (2) number of days with no trypanosomes, and (3) total therapeutic effect, which are all higher than that produced by 3 mg. of stibamine glucoside. Figs. 1 and 2 give the same information graphically; fig. 1 showing the percentage of animals surviving each day for each drug, and fig. 2 the average number of pluses for the whole group each day.

22. If, however, Table II is referred to again, it will be seen that the survival times overlap in the two groups of animals. Mouse No. 13 of the stibamine

glucoside group, for instance, did not die for 18 days, whereas mouse No. 7 injected with "urea stibamine" died on the fourteenth day. It is clear,

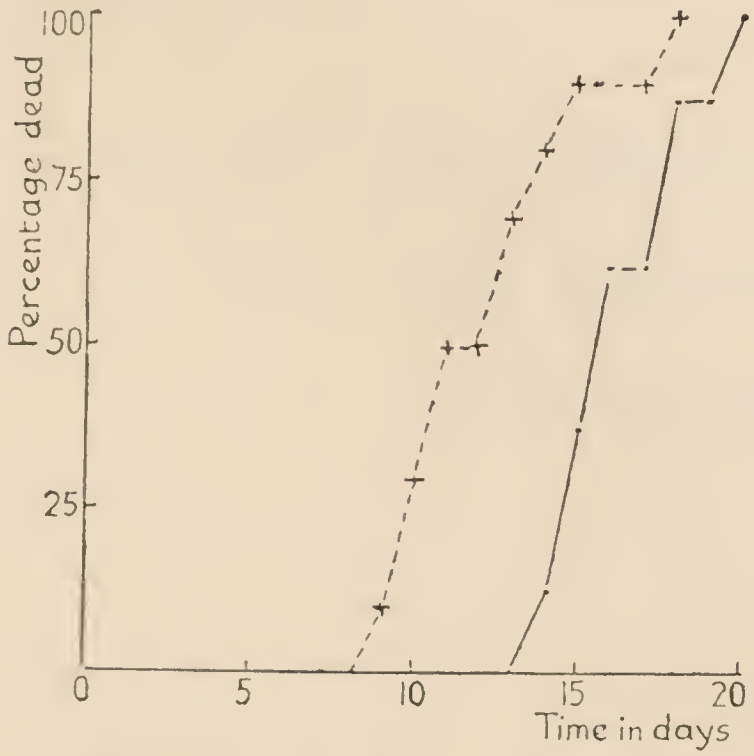

FIG. 1.—Percentage of mice dead on each day after injection of 1·5 mg. urea stibamine (B.926)—(full line) and 3·0 mg. stibamine glucoside W.3 per 20 g. mouse (dotted line).

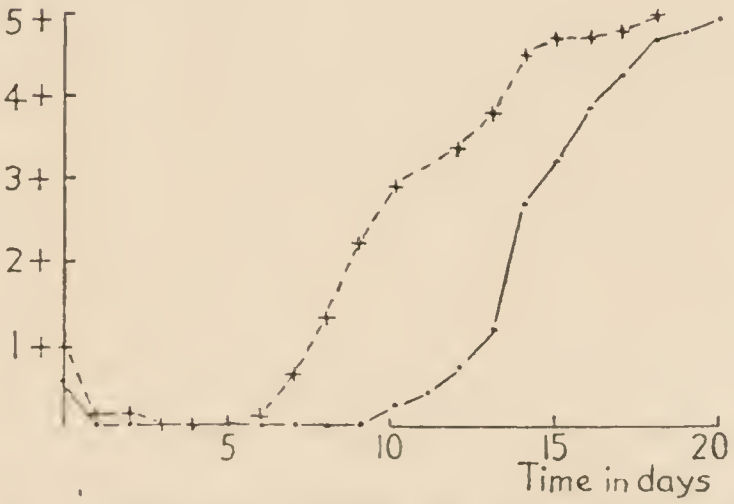

FIG. 2.—Average intensity of infection-number of pluses assigned daily—dead mice being reckoned as 5+. 1·5 mg. B.926 per 20 g. mouse (full line), 3·0 mg. W.3 per 20 g. mouse (broken line).

therefore, that there is a possibility that the difference in *mean* survival time is not due to a real difference in activity of the two drugs, but that the difference has arisen solely by chance amongst the animals used. To determine whether the differences shown in Table III are real differences, or merely random differences due to the variability of mice, we have applied simple statistical tests. The statistical method used is that developed by Fisher from "Students" work (Fisher, p. 109). The ratio of the difference of the average values,

Table III.

Method.	Drug.	Dose.	Mean.	No. of animals.	"t" (see below).	"P" (see below).
		mg.				
Survival time	" Urea stibamine " (B.926)	1·5	16·5	8	+3·57	$0{\cdot}0_3 6$
	Stibamine glucoside (W.3)	3·0	12·4	10		
Number of days with no trypanosomes	" Urea stibamine " (B.926)	1·5	12·1	8	+3·24	$0{\cdot}0_2 2$
	Stibamine glucoside (W.3.)	3·0	7·9	10		
Average therapeutic effect	" Urea stibamine " (B.926)	1·5	69	8	+3·34	$0{\cdot}0_2 1$
	Stibamine glucoside (W.3)	3·0	48·7	10		

obtained by either of the above methods, to the standard deviation of the difference, is estimated by the formula

$$t = (m_1 - m_2) \sqrt{\frac{(n_1 + n_2 + 2)(\Sigma d_1^2 + \Sigma d_2^2)}{(n_1 + 1)(n_2 + 1)(n_1 + n_2)}}$$

where t is the ratio of the difference of the means to the estimated standard deviation, $(n_1 + 1)$ is the number of animals in one group and $(n_2 + 1)$ the number in the other, Σd_1^2 is the sum of the deviations from the mean of the individual values for one group, and Σd_2^2 the same for the other group. For the experiment described for the mean survival time,

$$t = (16{\cdot}5 - 12{\cdot}4)/\sqrt{18 \times 96 \div (8 \times 10 \times 16)} = +3{\cdot}57$$

the number of animals $(n_1 + 1)$ and $(n_2 + 1)$ being 8 and 10 ; the sum of the squares of the deviations for the two groups being 96. In this and for all the succeeding experiments the convention has been adopted of subtracting the mean for the standard stibamine glucoside from that of the test group, so that, when the mean for the test group is greater than that for the standard, the sign is positive. The magnitude of the value of "t," irrespective of the sign, provides a means of judging whether the difference between two such means is statistically significant or not. For large groups of animals the difference is generally taken as being statistically significant if the value of "t" is more than 2·0, values larger than this value only occurring by chance in about 5 per cent. of trials ; but for small groups, such as those used, the same degree of significance is only obtained for values of "t" larger than 2. Tables have been published (*e.g.*, Fisher, p. 137) which give the probability "P" of certain values of "t" being exceeded by chance for any given number of animals. The values of "t" and "P" for the three methods of analysis of experiment

are given in Table III. Using Fisher's table, it is found that for this number of animals a value of 3·57 would only be exceeded by chance in less than one in a thousand trials ($P = < 0{\cdot}001$). This is to say that, unless 1·5 mg. of "urea stibamine" (B.926) is really more active than 3 mg. of stibamine glucoside, such a difference of mean survival times would not arise more than once in a thousand such comparisons as experiment 1; so that there is a high degree of probability that this drug is more than twice as active as stibamine glucoside in mouse trypanosomiasis. Similarly, the differences in the number of days with no trypanosomes would only arise by chance in about the same proportion of trials. The final value obtained is given in the next paragraph. The same treatment is applied to the method of evaluating these results by the number of days with no trypanosomes and by what we have called the "therapeutic effect." The survival time shows a slight advantage in the significance of the results, and this is the general rule (see Table X).

23. The next part of this section (Tables IV, V and VI) gives comparisons, based on survival time, of all the drugs we have tested. In the tables are given first the name of the compound with that of the standard with which it is compared immediately below. The next column gives the identification number of the compound used for cross reference to the chemical section. The third column gives the actual dose of the drug injected for each 20 g. of mouse, the fourth column gives the mean survival time of each group of mice, the fifth column the number of animals injected with each dose, and the sixth and seventh the values of "*t*" and "P" calculated as previously explained. In the last column is given the amount of the drug equivalent to 1 mg. of the glucoside; and this is either calculated from the ratio of the dose of the drug under test to that of this standard which, in any one comparative experiment, gives a statistically insignificant difference in the survival times of the two groups, by interpolation between two dose-ratios which give significant differences of opposite signs.

24. "*Urea Stibamine* (*Brahmachari*)."—Table IV, *a*, *b*, *c*, gives the comparisons based on survival time, of three experiments with "urea stibamine (Brahmachari)" (B.926). The drug was compared on one occasion with an equal weight of stibamine glucoside (Table IV, *a*), on another with twice its weight (Table IV, *b*, experiment already described), and on a third with three times its weight of stibamine glucoside (Table IV, *c*). When the ratio of the doses used is unity (Table IV, *a*) the value of "*t*" is 6, and such a value could only arise by chance errors of sampling in about one in 10 million such trials. It is certain therefore that this drug is considerably more active than stibamine

Table IV.—Therapeutic Action of various products, as estimated by Time of Survival after Injection of Drugs into Mice Infected with Trypanosomes. Each experiment includes the results on one group of mice treated with the drug to be tested and one group treated with the standard.

Drug.	Key No.	Dose.	Means of survival times.	No. of animals.	"*t*."	"P."	Dose equivalent to 1 mg. of glucoside.
		mg.					
a. "Urea stibamine (Brahmachari)"	B.926	1·5	11·8	8	+6	$0·0_41$	mg. 0·33
Stibamine glucoside	W.3	1·5	8·4	7			
b. "Urea stibamine (Brahmachari)"	B.926	1·5	16·5	8	+3·57	$0·0_36$	
Stibamine glucoside	W.3	3·0	12·4	10			
c. "Urea stibamine (Brahmachari)"	B.926	1·0	7·36	11	+1·01	0·32	
Stibamine glucoside	W.3	3·0	6·73	11			
d. "Urea stibamine (Brahmachari)"	B.1128	1·3	9·1	11	+2·13	0·04	0·29
Stibamine glucoside	W.3	4·0	6·3	9			
e. "Urea stibamine (Brahmachari)"	B.1128	0·75	7·58	12	−3·1	0·002	
Stibamine glucoside	W.3	3·0	8·6	10			
g. Alcohol-washed "urea stibamine"	B.3	0·75	13·0	11	+0·26	0·86	0·25
Stibamine glucoside	W.3	3·0	12·8	11			
h. Product containing *p*-carbamidophenylstibinic acid (urea preparation)	G.7	3·0	20·71	7	1·12	0·28	0·5
Stibamine glucoside	W.3	6·0	17·57	7			
j. Sodium carbamidophenylstibinate (cyanic acid preparation)	G.5	1·5	9·4	10	0·84	0·42	0·5
Stibamine glucoside	W.3	3·0	8·75	8			
k. Product identical with "urea stibamine"	G.12	1·0	12	13	+0·13	0·9	0·33
Stibamine glucoside	W.3	3·0	11·5	11			
l. Product containing *s*-diphenylcarbamide-4 : 4′-distibinic acid (urea preparation)	G.10	1·0	9·46	16	+3·34	$0·0_21$	0·25
Stibamine glucoside	W.3	3·0	8·66	12			
m. Product containing *s*-diphenylcarbamide-4 : 4′-distibinic acid (urea preparation)	G.10	0·75	5·54	11	+0·52	0·6	
Stibamine glucoside	W.3	3·0	5·18	11			
n. Product containing *s*-diphenylcarbamide-4 : 4′-distibinic acid (urea preparation)	G.11	1·0	11·0	11	0	—	0·25
Stibamine glucoside	W.3	4·0	11·0	11			
p. *s*-diphenylcarbamide-4 : 4′-distibinic acid (phosgene preparation)	G.9	1·4	16·4	9	+1·3	0·21	0·47
Stibamine glucoside	W.3	3·0	14	7			
q. *s*-diphenylcarbamide-4 : 4′-distibinic acid (phosgene preparation)	G.9	1·5	15·6	10	−1·6	0·12	0·6
Stibamine glucoside	W.3	3·0	23	9			

glucoside in mouse trypanosomiasis. When the ratio of the dose of drug to the dose of stibamine glucoside is reduced to 0·5 the value of "*t*" is still very high and "P" is proportionately small. When the ratio of the doses injected is 0·33 the value of "*t*" is 1·01 and the value of "P" is increased to 0·32. Owing to the inevitable variation amongst mice a value of "*t*" even greater than this might be obtained once out of three times, approximately, from a group of mice divided at random into two sets and treated with the same dose of one drug. We may therefore take it that the therapeutic action of 1 mg. of "urea stibamine (Brahmachari)" in mouse trypanosomiasis is equivalent to that of 3 mg. of stibamine glucoside. Table IV, *d, e*, gives the results based on survival times with a second sample of "urea stibamine (Brahmachari)" (B.1128), examined in the same way, and shows that the equivalent of 1 mg. of the glucoside is significantly less than 0·33 and significantly greater than 0·25 mg. We have taken it therefore as the geometric mean (0·29) of these figures. This sample is slightly more active than the previous one.

25. Table IV, *h*, gives the results of the therapeutic test of the product containing *p*-carbamidophenylstibinic acid (urea preparation) (G.7). This preparation has been discussed in the chemical section under § 13. The activity of this preparation in this infection is equal to that of twice the weight of stibamine glucoside.

26. *p*-carbamidophenylstibinic acid, prepared by means of cyanic acid (G.5) (see § 14), was tested in the form of the sodium salt, and the comparison is given in Table IV, *j*. The result shows that its activity is about twice that of stibamine glucoside but only about three-fifths of that of "urea stibamine." These relations have been confirmed in another experiment, reported later. The comparatively low trypanocidal activity of this substance confirms the chemical evidence that it is not the active constituent of "urea stibamine (Brahmachari)"; since the alcohol-washed material (B.3) with C : N = 7 (*loc. cit.*) is slightly more active than the original product (Table IV, *g*). The same substance, prepared by an alternative method (see footnote, p. 59), was found by Roehl to be inactive on kala-azar in the hamster (Schmidt, 1930).

27. As has been explained (§ 7), the active material is probably the di-substituted urea. Two samples of a product containing *s*-diphenylcarbamide-4 : 4′-distibinic acid (urea preparation) (G.10; G.11), prepared as described in § 27, were examined. The therapeutic results are given in Table IV, *l*, *m* and *n*, the first two referring to one sample and the last to the

other. 1 mg. of G.10 gives a significantly higher survival time than 3·0 mg. of stibamine glucoside, while the difference between 0·75 mg. of G.10 and 3·0 mg. of stibamine glucoside is not significant. The dose of this product which is equivalent to 1 mg. of stibamine glucoside is 0·25 mg. The sample G.11, which was a duplicate preparation, gives the same ratio.

28. Results with *s*-diphenylcarbamide-4 : 4′-distibinic acid, prepared by means of the action of phosgene on the amino-acid (§ 12) (G.9), are given in Table IV, *p* and *q*. The compound was not appreciably soluble in alkali ; comparison with the soluble products is therefore difficult. It was injected intraperitoneally in suspension in mucilage of acacia. When compared in this way (Table IV, *p*) 0·47 mg. was not significantly different in its action from 1 mg. stibamine glucoside injected intravenously. The value of "*t*" was +1·3. In another experiment (Table IV, *q*) 1·5 mg. of G.9 and 3 mg. of stibamine glucoside were both injected intraperitoneally, the value of "*t*" then being −1·6, suggesting a slightly larger ratio, but the difference is still not significant. The second experiment is shown in fig. 3 as percentage of

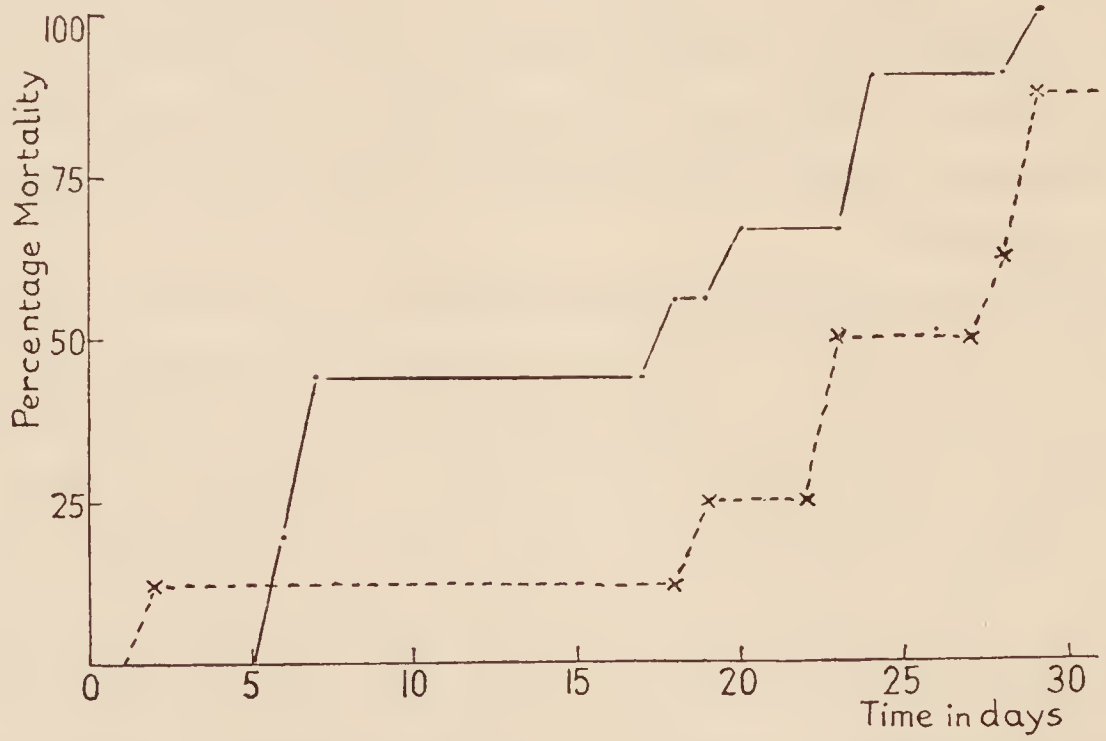

Fig. 3.—Percentage mortality produced in two groups, one injected with 6 mg. G.9 (full line) and the other with 12 mg. of stibamine glucoside W.3.

survivals on each day after injecting the drugs. It will be seen that during the first few days the insoluble drug was less effective than the soluble one, but those that survived the first few days lived practically as long as those injected with stibamine glucoside. The mice were fairly heavily infected before the drugs were administered, and apparently the rate of solution of the insoluble compound was so slow at first, that the infection proceeded to a fatal outcome in some of the animals before a sufficiently high concentration of the drug was

present to inhibit the development of the infection. The dose-equivalent of this substance, with respect to stibamine glucoside, is about 0·5, whereas the soluble preparation containing it, made by means of urea, has a dose-equivalent of 0·25. The fact that one of the two is so sparingly soluble, however, detracts from the value of the comparison.

29. Table IV, *k*, gives the results obtained with the product of the action of urea on partially hydrolysed *p*-acetylaminophenylstibinic acid (G.12) (§ 10). The equivalent dose is the same as that of the commercial "urea stibamine," and the product, as already pointed out, is similar to the commercial material in other ways.

30. Table V gives the results of the comparison of stibanilic acid with stibamine glucoside. Both in the form of the sodium and of the ammonium salts

Table V.

Drug.	Key No.	Dose.	Means of survival times.	No. of animals.	"*t*."	"P."	Dose. equivalent to 1 mg. of glucoside.
		mg.					mg.
. Sodium *p*-aminophenylstibinate (90° preparation)	G.1	3·3	17	8	+3·87	0·0001	
Stibamine glucoside	W.3	6·0	12·7	11			
. Sodium *p*-aminophenylstibinate (90° preparation)	G.1	1·0	8·0	11	−1·6	0·13	0·4
Stibamine glucoside	W.3	3·0	10·9	10			
. Sodium *p*-aminophenylstibinate (90° preparation)	G.1	0·75	9·7	13	−3·26	0·0001	
Stibamine glucoside	W.3	3·0	14·1	9			
. Sodium *p*-aminophenylstibinate (90° preparation). Kept for 3 months at 37°	G.2	1·5	9·4	11	−0·22	0·85	0·5
Stibamine glucoside	W.3	3·0	9·625	8			
. Ammonium *p*-aminophenylstibinate (90° preparation)	G.1	1·2	8·0	10	−0·83	0·42	0·4
Stibamine glucoside	W.3	3·0	8·57	7			

the equivalent dose of the amino-acid is 0·4 mg. A sample of the acid kept in sealed tubes at 37° for 3 months gave a sodium salt with an equivalent dose of 0·5 mg. This is probably significantly different from the original figure, especially as another sample prepared from a different sample of stibacetin showed a similar deterioration.

31. Table VI gives the results of some experiment with stibacetin (W.1), which was the batch used for the preparation of the compounds referred to up to this point. 3·0 mg. is not significantly more active than 4·0 mg. of

Table VI.

Drug.	Key No.	Dose.	Means of survival times.	No. of animals.	"*t*."	"P."	Dose equivalent to 1 mg. of glucoside.
		mg.					mg.
a. Stibacetin	W.1	3·0	19	9	1·50	0·2	
Stibamine glucoside	W.3	4·0	15	10			0·7
b. Ammonium salt of *p*-acetylaminophenylstibinic acid	G.14	2·5	13·15	8	+3·0	0·01	
Stibamine glucoside	W.3	3·0	8·8	5			0·7
c. Ammonium salt of *p*-acetylaminophenylstibinic acid	G.14	2·0	10·3	10	−2·2	0·045	
Stibamine glucoside	W.3	4·0	13·0	8			

stibamine glucoside, but since the value of "*t*" is as much as 1·5, 0·7 mg. is probably nearer the true equivalent of 1 mg. stibamine glucoside than 0·75. The ammonium salt of this preparation has an equivalent dose indistinguishable from this (Table VI, *b* and *c*). Another sample of stibacetin (W.2) had somewhat less activity, 0·9 mg. being equivalent to 1 mg. of stibamine glucoside. A series of derivatives prepared from this sample were all less active than those from W.1 (see Table VII). The difference in therapeutic activity persists throughout the series, and although the difference is of an order which is barely significant in each case (see later) yet the fact that it occurs in the same direction for five different pairs of compounds renders it highly probable that the difference is a real one, although there was no corresponding difference

Table VII.

	Equivalent dose when made from	
	Stibacetin W.1.	Stibacetin W.2.
	mg.	mg.
Ammonium salt of *p*-acetylaminophenylstibinic acid	0·7	—
Sodium salt of *p*-acetylaminophenylstibinic acid	—	0·9
Sodium *p*-aminophenylstibinate	0·4	0·6
Sodium *p*-aminophenylstibinate. Kept at 37° for 3 months	0·5	0·66
Sodium *p*-carbamidophenylstibinate (cyanic acid preparation)	0·5	0·6
Product containing *p*-carbamidophenylstibinic acid (urea preparation)	0·5	1·0

in composition between the members of the pairs. This point seems to us to be of considerable importance, as showing that more remains to be done before the biological control of the preparation of organic antimonials can be dispensed with.

32. *Accuracy of Therapeutic Test.*—The necessity for using a standard on a comparison group in each experiment is shown by many of the experiments, two examples of which are given in Table VIII. The degree of infection in each case was approximately the same, but the experiments compared were done at different times. The first column gives the dose of stibamine glucoside used. 3 mg. gave a survival time of 12·1 days at one time and 7·4 at another. The values of "*t*" and "P" worked out in the same manner as before are given, and it will be seen that there is a significant difference. Similarly, 1·5 mg. produced in two different experiments survival times of 5·7 and 8·4 days, which are again significantly different. The difference was not due to differences in degrees of infection in the experiment, for the following reason. An experiment was done later in which the initial infection was less than in the

Table VIII.—Showing Significant Differences between the Effects of the same Dose of Stibamine Glucoside Injected at different times.

Dose.	Mean survival times.		"*t*."	"P."
	1st experiment.	2nd experiment.		
mg.				
3	12·1	7·4	2·02	0·05
1·5	5·7	8·4	2·68	0·02

above experiment by a degree which was quite clearly distinguishable, even by the rather crude method of enumeration adopted, and yet the survival time after 1·5 mg. of stibamine glucoside was 8·3 days as compared with 8·4; so that lowering the degree of infection does not always produce a difference in survival time. There seemed to be some evidence that the virulence of the infection was increasing during the series of experiments, perhaps on account of rise of room temperature associated with summer weather.

33. On some occasions the group of mice injected divided itself into two, one part of the group dying early with no disappearance of trypanosomes, and the other part losing trypanosomes, if the drug was active enough, and surviving very much longer. Some of these antimony preparations, at any rate, seem

to have a latent period of action, and, if the infection is very great before this latent period is passed, the infection becomes heavy enough to kill the animal before the drug can take effect.

34. The error of these comparisons can be fairly adequately estimated from an experiment put up especially to determine this point, and from evidence which can be derived from the various repetitions of the experiment on the same drug, already recorded. In the experiment designed to test this point, two groups of 12 and 10 animals were injected respectively with 3 mg. and 4 mg., irrespective of body weight. The results are given in Table IX. Tested by

Table IX.

Drug.	Key No.	Dose.	Means of survival times.	No. of animals.	"*t*."	"P."	Dose equivalent to 1 gm. of glucoside.
		mg.					
Stibamine glucoside	W.3	4	20·1	12	+2·6	0·016	—
,, ,, ...	W.3	3	12·1	10			

all three methods there was a significant difference between these two doses. The failure to adjust to the body weight necessarily increases the standard deviation, because the heavy mice will get too little, and the small mice too much, so that we actually get a lower estimate of the significance of the difference ; and it may be taken as fairly certain that doses differing in therapeutic activity in the ratio of 1 to 1·33 can be distinguished, when two groups of 10 animals each are used for comparison, and the results are confirmed by the following evidence.

35. For two drugs the ratio of the dose of the drug to that of the standard was the same in two separate experiments, and the results for both drugs were not significantly different from that of the standard on both the occasions on which they were tried. So far as it goes, therefore, the repetition of the experiment gives the same result, although in both these cases the degree of infection for the two corresponding experiments was quite different.

36. On four occasions the drug was injected in the first experiment in a dose bearing a ratio to the standard of half that which it had in the second experiment. In one the value of "*t*" for the larger dose was +6, for the smaller dose +3·57 ; in another "*t*" was +1·8 for the larger and −2·7 for the smaller dose ; for the third "*t*" was +3·7 and −4·3 for the larger and smaller doses

respectively ; in the fourth "t" was —0·235 for the larger and —4·6 for the smaller. So that one may expect, on the average, that, if the doses are appropriate, a dose of 2 units will be significantly greater in effect than a given quantity of a standard, and a dose of 1 unit significantly less. That is to say, the ratio which can be distinguished is not greater than 1·414 (the geometric mean of 1 and 2), since the values of "t" in general are more than sufficient (2·1 for a value of "P" of 0·05) to call the difference significant. This is borne out by further experiments in which the ratios of doses were smaller. In one experiment 1 mg. "urea stibamine" in one case and 0·75 mg. in the other were compared with 3 mg. of stibamine glucoside. In the first case the value of "t" was —1·6, in the second —3·6. The discrimination is therefore of some ratio less than 1·33. In another set of experiments (experiments 16 and 17), in the first case "urea stibamine" was shown to be significantly more active on trypanosomiasis in the mouse than three times its weight of stibamine glucoside, "t" being +2·13, and in the second case to be significantly less active than four times its weight, with a "t" of —3·1. The distinguishable ratio indicated in this case is 1·15. That is to say, if all experiments behaved as this one has done, it would be possible to distinguish, on two groups of 10 animals, two similar antimony compounds differing in activity by 15 per cent. ; but this estimate is probably unduly favourable. As an approximation, it may be taken that the therapeutic activity assigned as the result of these experiments is on the average not in error by more than 30 per cent., possibly not more than 25 per cent. For the purpose of this paper these limits may be taken as sufficient. To work out accurately the average error would mean a large series of experiments with the same drug, and would only be of interest in connection with the routine standardisation of these and similar compounds intended for therapeutic use.

B.—*Toxicity Tests.*

37. A number of the compounds were examined for toxicity on mice. The drugs were made up in distilled water to such a volume that the required dose was contained in 0·5 c.c. for 20 g. of mouse, the volume being adjusted to the weight of the animal. Distilled water was used because some of the compounds gave cloudy solutions with saline. Comparison of stibamine glucoside in distilled water and saline showed no difference in toxicity. On the average about 150 mice were used in groups of 30 to 50 at different doses for each drug. The doses used were chosen from preliminary experiments so as to fall in the range between doses causing no deaths and those causing the death of all animals injected. In this way a characteristic toxicity curve (Trevan, 1927)

for the drug on mice has been obtained for the more important of the preparations already dealt with.

38. Fig. 4 shows the characteristic curve for stibamine glucoside, for which

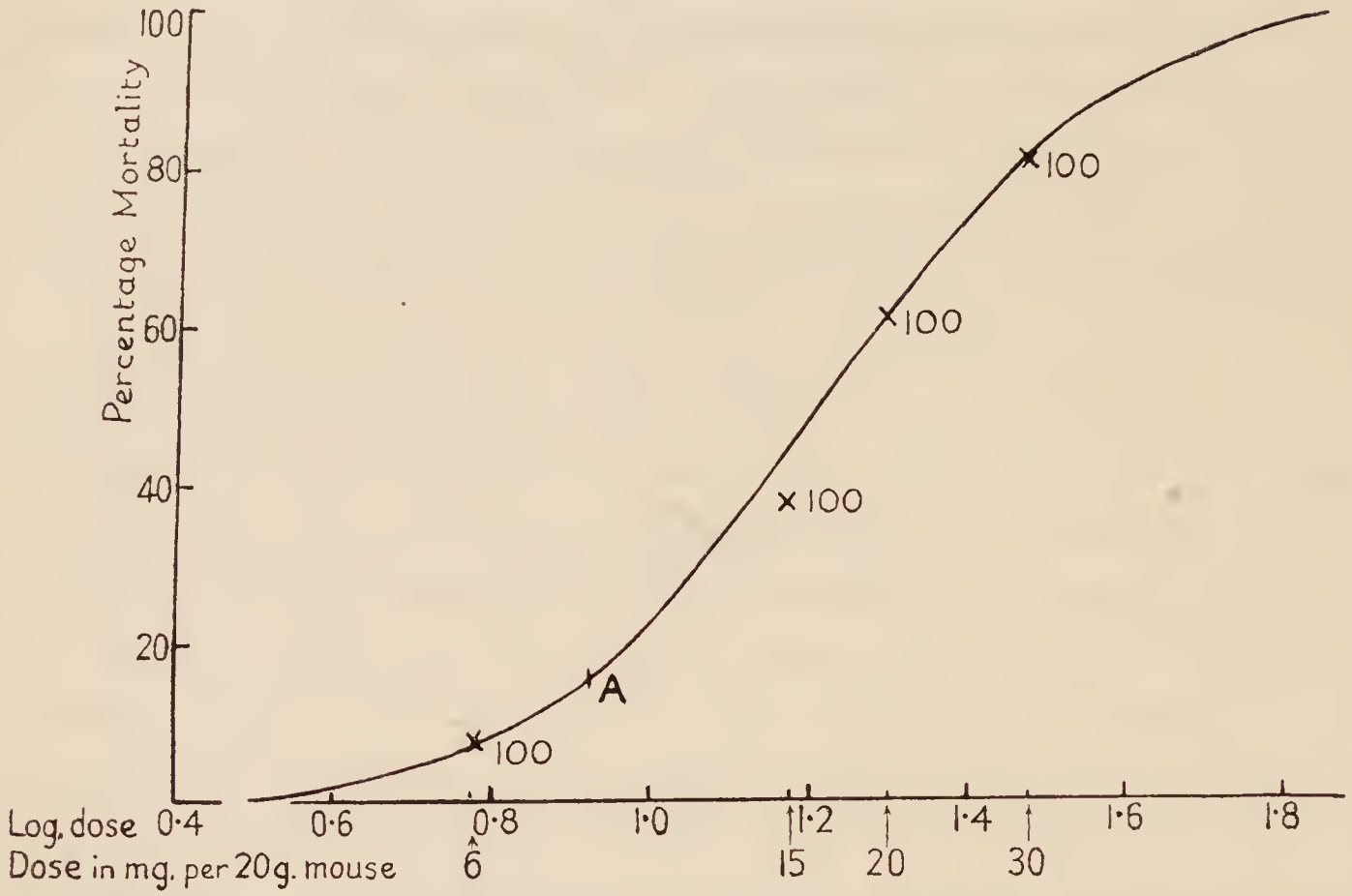

Fig. 4.—Characteristic toxicity curve for stibamine glucoside. Ordinates percentage mortality abscissæ logarithm of dose and dose in milligrams per 20 gm. mouse. Numbers at each point represent number of mice used.

400 mice were used. Percentage mortality is plotted against the logarithm of the dose injected. The number of animals injected is indicated by the figure attached to each observed point. The smooth curve is a normal percentile frequency curve ; it was fitted in the following manner. A straight regression line was fitted to the observed point in the standard manner. The 50 per cent. mortality ordinate of this line is taken as given the mean lethal dose (the LD 50), and a series of percentile frequency curves of different slopes was then drawn through this point until one was found from which the sum of the squares of the deviations of the observed points was a minimum. The apparatus described by Trotter (1923) for the graphic fitting of curves was found very useful for this purpose. It will be seen that the frequency curve is a very good fit for the observed points. It has been found that if logarithms of doses are plotted againt mortality in this way a large number of drugs give a characteristic which is indistinguishable from a normal frequency curve. One of us has previously pointed out (Trevan, 1927) that the shape of these characteristics depends on the distribution of variability of the " individual lethal dose,"

Table X.

Reference to table.	Survival time.	Number of days with no trypanosomes.	Average therapeutic effect.
3*b*	+3·57	+3·24	+3·34
3*c*	+1·01	+0·4	+1·07
3*d*	+2·13	+1·1	+1·55
3*e*	−3·1	−2·06	−2·5
3*g*	+0·26	−0·37	—
3*h*	−0·43	−1·43	−0·705
3*j*	−2·05	−1·5	−1·9
3*k*	+0·13	−0·60	−0·27
3*l*	+1·86	+2·56	+2·3
3*m*	+0·52	−0·62	+0·51
3*n*	0	+1·73	+0·52
3*p*	+1·6	+1·1	—
4*a*	−3·45	+0·32	—
4*b*	−1·86	−0·7	−3·16
4*c*	−1·59	−1·56	−1·83
4*d*	+0·49	+0·71	+1·0
4*e*	−2·40	−3·0	−1·8
4*g*	+3·87	+3·55	+1·46
4*h*	−1·6	−2·4	−1·97
4*j*	−3·26	−3·4	−3·65
4*k*	−0·22	+0·66	—
4*l*	−0·83	+1·03	—
4*t*	−0·09	−0·45	−0·74
5*b*	+0·32	−0·18	0
5*c*	−2·4	+2·08	—
5*e*	−4·63	−5·95	−5·2
5*f*	−3·2	−2·24	−2·8
5*g*	−1·62	−1·42	—

which is the dose just sufficient to kill each separate member of the groups of mice injected. Thus, for example, in the group injected with a dose of which the logarithm is 0·789 (6 mg.), 8 per cent. were killed. That is to say, of the group of animals injected with this dose 8 per cent. had an " individual lethal dose " of less than 6 mg., and 92 per cent. required more. In the same way, 61 per cent. of animals in the group injected with 20 mg. have an individual lethal dose less than 20 mg., and 39 per cent. greater. Consequently, by graphically differentiating the characteristic curve, close approximations to the distribution of the logarithms of the individual lethal doses would be obtained, and would in this case be the usual bell-shaped normal frequency curve, with the mean at a dose of 16·2 mg. (the logarithm of which is 1·21)—which is the same, for a symmetrical curve, as the median dose producing 50 per cent. mortality (the LD 50).

39. The mean dose is the dose which represents the dose necessary to kill the " average " mouse. What is generally quoted in the literature for organo-metallic compounds is the " maximum tolerated dose," by which is apparently

implied the dose which kills very rarely. In general, no quantitative interpretation of "very rarely" is given, and the attempt is made to determine directly the dose which kills an occasional mouse, when successive groups of 2 to 10 animals are injected with increasing doses. It is obvious that, apart from the general uncertainty arising from the use of small groups of animals, the dose at which the first death occurs will, on the average, be larger the smaller the group of animals used, for the relative frequency with which any dose will give one or more deaths in a group of n animals is $1 - P^n$, where "P" is the probability of survival with this particular dose, and P^n the probability that all will die. Since "P" is always less than 1, $1 - P^n$ is larger the greater the value of "n," so that for groups of animals injected with increasing doses, the dose which first gives a fatality is likely to be higher when a small group is used, as well to have a larger range on repetition. An estimation of the "maximum tolerated dose," which is not only subject to large chance errors, but depends in a special way on the size of the group injected, seems to us to be fundamentally unsound; and we would propose the following procedure as more logical and having a less ambiguous interpretation. If a variable, such as the logarithm of the individual lethal doses, is distributed in a normal manner, the best estimate of the frequency of the occurrence of any deviation from the average is obtained by calculating its ratio to the standard deviation of the variable, and using the known tables of the frequency of deviation in terms of multiples of the standard deviation. By this means the "maximum tolerated dose" can be defined as the dose which produces an appropriately small percentage mortality, and its estimation will be affected only by an error diminishing with the number of animals used, and distributed normally around the true limiting value of the particular dose chosen. For a normal curve 15·866 per cent. of deviations are less than the mean minus the standard deviation and 15·866 per cent. greater than the mean plus the standard deviation. So that the standard deviation of the logarithms of individual lethal doses can be determined graphically from the characteristic by finding the logarithm of the dose which gives 15·866 per cent. mortality (A, fig. 4) and subtracting it from the logarithm of the dose causing 50 per cent. mortality. For stibamine glucoside the logarithm of the dose causing 15·86 per cent. mortality is 0·92, point S on curve, and the standard deviation is $(1·21 - 0·92) = 0·29$.

The next point to decide is, what multiple of the standard deviation is to be subtracted from the mean to arrive at a reasonable criterion of the "maximum tolerated dose." The choice must be more or less arbitrary, but, once

arrived at, it will render the comparison between the results of different workers of more value. We suggest that the maximum tolerated dose should be taken as the dose which kills approximately 1 in 742 of mice injected. This odd figure is chosen because it corresponds to an integral multiple of the standard deviation, and the dose giving this mortality is obtained by subtracting three times the standard deviation of the logarithms of the individual lethal dose from the logarithm of the median dose. We would suggest that the abbreviated LDO be used for this dose. It is of course, to be clearly distinguished from the Lo used in the titration of diphtheria antitoxin. For stibamine glucoside three times the standard deviation is 0·87 and the logarithm of the maximum tolerated dose as defined above is $1{\cdot}21 - 0{\cdot}87 = 0{\cdot}34$; the maximum tolerated dose is therefore 2·19 mg.; one in 740 mice will be killed by a dose about 2·19 mg. The estimate suffers from an uncertainty, due to the ordinary random errors of sampling of mice, but is, we think, preferable to the usual method, which suffers not only from these errors but from random definition also.

40. Table XI gives the median lethal dose (column 2), the maximum tolerated dose LDO as defined above (column 3) and the standard deviation of the logarithm of the dose (column 4). The average toxicity as judged by the median lethal dose is not widely different except for stibamine glucoside which is less than half as toxic as the next (stibacetin) and is about a third of the toxicity of the aminophenylstibinic acid. The masking of the amino-group by glucose in stibamine glucoside is presumably the cause of this low toxicity.

Table XI.—Toxicity for Mice.

	No. of animals.	Median lethal dose, mg. per 20 g. mouse.	LDO, mg. per 20 g. mouse.	Standard deviation log. dose.	Comparative therapeutic value.
Stibacetin (W.2)	165	7·69	2·02	0•167	2·70
Stibacetin (W.1)	115	6·49	1·93	0·175	2·76
Sodium *p*-aminophenylstibinate (G.1)	150	4·95	1·38	0·185	3·45
Sodium *p*-aminophenylstibinate (G.3)	131	5·37	0·478	0·354	0·96
" Urea stibamine " (B.1128)	167	6·31	1·20	0·24	4·13
Product identical with " urea stibamine " (G.12)	84	6·76	2·24	0·16	6·79
Product containing *s*-diphenylcarbamide 4 : 4′-distibinic acid (G.10)	127	4·27	0·933	0·22	3·73
Product containing *s*-diphenylcarbamide 4 : 4′-distibinic acid (G.11)	157	3·98	0·872	0·22	3·49
Stibamine glucoside (W.3)	400	16·2	2·19	0·29	2·19
Diethylamine antimonate (G.15)	224	5·37	1·55	0·18	—

A similar but less marked diminution in toxicity is brought about by the insertion of an acetyl-group in *p*-aminophenylstibinic acid (compare G.1 and stibacetin W.1 from which this was prepared). The difference between these is probably statistically significant. A similar difference is to be observed between the LD 50 of stibacetin W.2 and that of *p*-aminophenylstibinic acid G.3 which was prepared from it.

41. The product containing *s*-diphenylcarbamide-4 : 4′-distibinic acid (urea preparation) (G.10, G.11), on the other hand, has as high a toxicity as the amino-acid and the therapeutic activity is still more enhanced. The corresponding product made from partially hydrolysed acetyl-derivative (G.12) as well as "urea stibamine (Brahmachari)" on the other hand, are both of the same order of toxicity as stibacetin. This could be accounted for by assuming that G.12 contains a substance with a lower toxicity than that of stibacetin, resulting from the action of urea on *p*-acetylaminophenylstibinic acid. As stated in § 16, however, this seems very improbable, and we cannot at present suggest a cause, unless the difference in toxicity is due to a difference in physical condition, an explanation we advance with some reluctance.

42. The figures for the standard deviation of the logarithm of the dose show some curious anomalies. The standard deviation varies from 0·18 (diethylamine antimonate) to 0·354 (*p*-aminophenylstibinic acid, G.3) for the different drugs. This difference is certainly a significant one. The dose just sufficient to kill individual mice therefore varies very much more for *p*-aminophenylstibinic acid than for diethylamine antimonate. The dose of the former acid just sufficient to kill about 1 in 740 mice (the LDO, see Table XI) is 0·478 mg.; of the latter, 1·55. The dose, on the other hand, which will *fail* to kill only 1 in 740, which we will call the LDC, and which can be estimated by a method analogous to that used for LDO, is 18·6 for diethylamine antimonate and 62 for amino-acid already referred to (G.3). The last figure seems at first sight ludicrous, but examination of fig. 5 shows that it is the inevitable deduction from the toxicity curve as determined, and a similar but less extensive spread is shown by stibamine glucoside (2·19 to 120 mg.), which is based on a much larger number of animals. The LDO of the amino-acid is therefore considerably lower than that of diethylamine antimonate, whereas the LDC is very much higher. The standard deviations of the logarithm of the dose can be divided roughly into three classes (1) those about 0·16 to 0·18, namely, stibacetin, one of the amino-acid preparation, diethylamine antimonate, and the product G.12 (see tables); (2) those from 0·22 to 0·24, the products of G.10, G.11 and B.1128; and (3) stibamine glucoside (0·29). The cause of

this difference in the variability of the response of the mice is obscure. In the last column we have given a comparative "therapeutic index" based on the

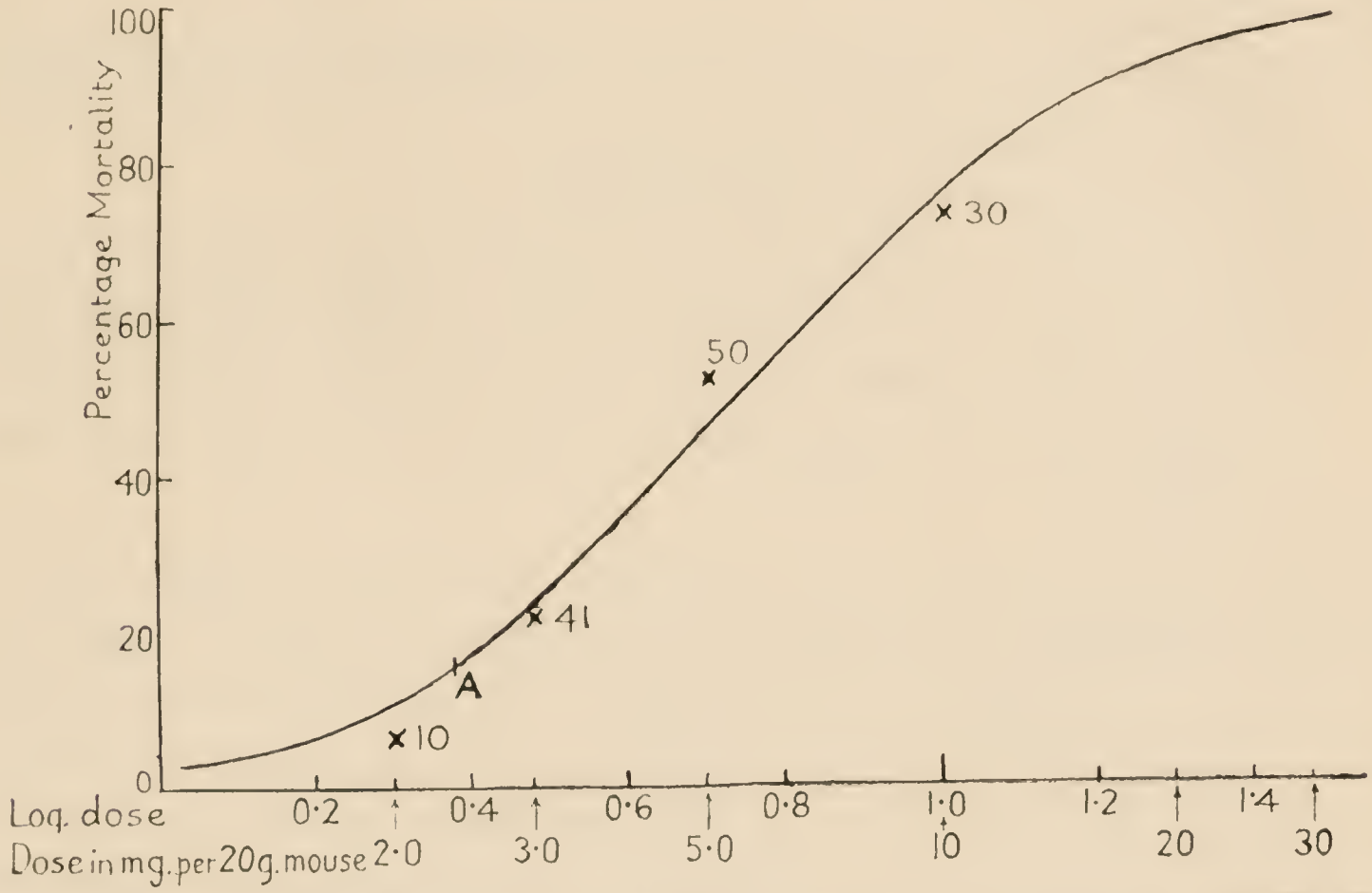

FIG. 5.—Characteristic toxicity curve for amino-acid G.3.

therapeutic effect of 1 mgm. of stibamine glucoside as the standard. The therapeutic index is calculated by dividing the LDO by the dose of the particular drug which is equivalent to 1 mg. of stibamine glucoside in therapeutic effect. The relative value obtained for the ratio between toxicity and therapeutic effect of two drugs must depend on the particular dose selected as representing the maximum tolerated, when the toxicity curves for the two drugs differ in slope. For example, we will take the two stibanilic acids G.1 and G.3. If we had chosen the LD 50 (median lethal dose) as the maximum tolerated dose, the therapeutic value would be $4{\cdot}95/0{\cdot}4 = 12{\cdot}75$ for G.1 and $5{\cdot}37/0{\cdot}5 = 10{\cdot}74$ for G.3—practically equal instead of in the ratio of 3·5 to 1; and if we had felt that a chance of one mouse in 730 dying was too high to call the dose producing that result the maximum tolerated dose (although it is probably a more stringent requirement than is usual), and decided that four times the standard deviation should be used for the calculation of the maximum tolerated dose, the advantage of G.1 over G.3 would have been increased. Again, the increase of the therapeutic index of the urea preparation G.12 over "urea stibamine" B.1128 (Table X) is almost entirely due to the smaller variability of response of mice to the former drug.

Summary.

Urea, in aqueous solution, is found to act upon *p*-aminophenylstibinic acid in two ways :—

(1) With hydrolysis at the bond between antimony and carbon and the formation of antimonic acid. This can occur in the cold, as well as above the temperature of rearrangement of urea.

(2) With formation of the mono-substituted urea, *p*-carbamidophenylstibinic acid, or the di-substituted urea, *s*-diphenylcarbamide-4 : 4′-distibinic acid, according to the conditions of the experiment and the quantities of the reacting substances chosen. This occurs between 75° and 100° C.

These two ureides have also been prepared by the action of cyanic acid and phosgene, respectively, upon *p*-aminophenylstibinic acid, and the second has been shown, by comparison of the therapeutic action of all these products upon a *Trypanosoma equiperdum* infection in mice, to be the protozoicidal constituent of the drug known as "urea stibamine," which was stated by Brahmachari to consist of the ammonium salt of *p*-carbamidophenylstibinic acid ; the latter is found to be considerably less active, so that it is important to fix the conditions of the reaction with urea, so as to obtain the di-substituted urea. As available in commerce, "urea stibamine" is found to contain, in addition to the di-substituted urea, antimonic acid, as mentioned above, together with some *p*-acetylaminophenylstibinic acid, resulting from incomplete hydrolysis of the stibacetin used as the starting substance ; owing to the weakly acid character of these materials, they retain only a small amount of ammonia, which suffices, in consequence of their predominantly colloidal nature, to bring the whole into solution.

The accuracy of comparison of therapeutic activity on trypanosomiasis in the mouse is discussed. It is shown that the comparison of survival times after treatment with these drugs is at least equal in value to any other method. The toxicity of a number of the products has been determined, using for this purpose a larger number of animals than has usually been employed, and characteristic toxicity curves obtained. The "maximum tolerated dose" is rigidly defined, and is chosen as the dose which kills 1 in 742 of the mice. Comparative therapeutic values are obtained by dividing the maximum tolerated dose of each drug, as so defined, by the dose which is equivalent in trypanocidal action to the standard dose of the reference substance, stibamine glucoside.

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